Point of View

Viral interference of the bacterial RNA metabolism machinery

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Abstract

In a recent publication, we reported a unique interaction between a protein encoded by the giant myovirus phiKZ and the *Pseudomonas aeruginosa* RNA degradosome. Crystallography, site-directed mutagenesis and interactomics approaches revealed this 'degradosome interacting protein' or Dip, to adopt an 'open-claw' dimeric structure that presents acidic patches on its outer surface which hijack two conserved RNA binding sites on the scaffold domain of the RNase E component of the RNA degradosome. This interaction prevents substrate RNAs from being bound and degraded by the RNA degradosome during the virus infection cycle. In this commentary, we provide a perspective into the biological role of Dip, its structural analysis and its mysterious evolutionary origin, and we suggest some therapeutic and biotechnological applications of this distinctive viral protein.

Introduction

The relationship between bacteria and the viruses that prey upon them is complex and ever evolving. Although studied intensively, discoveries continue to be made of different strategies employed by both virus and host to aid or evade infection, respectively. Bacteriophages have evolved multiple and varied mechanisms to efficiently infect their bacterial hosts. An ubiquitous strategy utilized by phages during infection is the production of proteins that modulate or redirect the functionality of specific host proteins.[1][2] From a bacteriophage perspective, these interactions are often crucial to evade the multitude of bacterial defense mechanisms or to alter the host metabolism in order to ensure an efficient infection cycle. The discovery of phage effector proteins that target regulatory hubs of the host bacterium could open new doors towards drug discovery and design.[3] The best studied example of such a hub is the RNA polymerase which is targeted by several phages at different interaction sites, influencing transcription by a wide range of mechanisms.[4] Another key regulatory hub is the RNA degradosome, a multiprotein complex responsible for RNA turnover and posttranscriptional gene regulation in bacteria. A general model for the *Escherichia coli* RNA degradosome has been described, with a core complex comprising the hydrolytic endonuclease RNase E, a phosphorolytic exoribonuclease, PNPase, the ATP dependent helicase RhlB, and a glycolytic enzyme, enolase.[5] However, the exact makeup and variability of the complex in important bacterial pathogens like *Pseudomonas aeruginosa* was not previously characterized.

Intracellular levels of any RNA are balanced by both synthesis and degradation, and must be well synchronized with cellular processes. As such, the degradation rate of individual RNAs is an important aspect of the control of gene expression. In bacteria, mRNA has a half-life of only 2 to 3 minutes, which allows the cell to quickly adapt to alterations in the environment and govern stress responses.[6][7] Therefore, we reasoned that an important regulatory hub such as the RNA degradosome would be a potential candidate for targeting by phage effector proteins, thereby disrupting this level of cellular control. The identification of such phage proteins had previously been limited to two examples, a phosphorylation-based inhibitor (Protein kinase 0.7, phage T7) that selectively stabilizes phage transcripts and an RNA degradosome activator from coliphage T4 (Srd), which has been found to destabilize host mRNAs.[8][9] In our recent publication[10], a phage effector protein was identified, encoded by the giant *Pseudomonas* phage phiKZ, able to specifically target the RNA degradosome of *P. aeruginosa*. This 'degradosome interacting protein' (Dip) was

shown to act by inhibiting the activity of the host ribonuclease RNase E. Additionally, the methods used to identify Dip also shed further light on the protein composition of the *P. aeruginosa* RNA degradosome. In this point-of-view commentary, we expand on this interaction and reflect on the impact of identifying this inhibitor of RNase E and understanding the mechanism of Dip.

The composition of the *P. aeruginosa* RNA degradosome

The strategy to identify phage-encoded proteins that interact with bacterial host proteins was based on the pull down of bacterial proteins (and/or complexes) during the early phase of a phage infection cycle.[11] By performing affinity purifications on *P. aeruginosa* cells containing a *Strep*II-tagged RNase E, Dip was identified in interaction with the RNA degradosome during phiKZ-infection.[10] Moreover, this and pull-downs using six other, unrelated *Pseudomonas* phages provided information on the composition of the *P. aeruginosa* degradosome itself for the first time (Figure-1A and Supplementary Table 1). The exoribonuclease PNPase co-purified with RNase E following infection with all used phages and was present in the pull-down experiment using heterologously expressed Dip and wild type *Pseudomonas* cell lysate. In this Dip-based pull down the RNA helicase DeaD was detected as well. Remarkably, during infection with the different phages, one to three different DEAD-box RNA helicases (RhlB, RhlE and DeaD) were co-purified with RNase E. Moreover, protein chaperone DnaK, which has previously been identified in complex with the RNA degradosome in *E. coli* [12][13], was co-purified during some phage infections. These findings indicate that the composition of the RNA degradosome may vary in response to different phage infections and might suggest that some phages possess more indirect mechanisms to affect the RNA degradosome as well.

Enolase could not be identified in any of the pull-down experiments, suggesting that this canonical component of the *E. coli* RNA degradosome does not form part of the *P. aeruginosa* complex, even though enolase is predicted to be present in the *P. aeruginosa* cytoplasm.[14] Finally, given the presence of ATP synthase and NADH quinone oxidoreductase (NuoD) and in these experiments, it is tempting to speculate that the list of metabolic enzymes capable of binding to the RNA degradosome in different bacterial organisms can be expanded. However, whether these proteins are genuine components of the degradosome assembly in *P. aeruginosa* remains to be established.

The functional role of Dip during phage infection

Having identified the RNA degradosome as a target of Dip, the question arose as to the functional consequences of this interaction. The role of Dip could be inferred from its *in vitro* inhibition of RNase E mediated cleavage of RNA substrates. This inhibitory effect was found on substrates of both bacterial and viral origin, indicating a lack of any specificity towards RNA substrates. Additionally, we found that the Dip protein reaches detectable levels in *P. aeruginosa* 9 minutes post infection, which was in agreement with a previously published RNA-seq analysis of phage phiKZ-infected *Pseudomonas* cells.[10][15] Since the protein remains present in the cells during the remaining infection cycle, a time-regulated mechanism by which the phage subverts the role of the RNA degradosome in transcript degradation and processing is suggested. It can be speculated that a delay between initial infection and Dip production allows for RNase E mediated degradation of host RNAs prior to the inhibition of this enzyme. In addition, the stabilization of the viral RNA during the middle and later stages of the phage infection cycle is consistent with the fivefold increase in cellular RNA levels during late infection stages.[15] In contrast, coliphage T4 uses a different strategy, since it over-activates the host RNase E with Srd, increasing degradation of host RNA during early infection stages.[9] The importance of Dip for efficient infection of *P. aeruginosa* by phiKZ remains to be established, but it is apparent from comparative genomics analyses that this protein does not share sequence homology to proteins of other (closely) related phage. In addition, the unique fold of this protein raises the question of its evolutionary origin and could support the observations that phiKZ forms a distinct branch of the *Myoviridae* family.[16]

Towards a structure-based interaction model for Dip and the RNA degradosome

Dip forms a dimer that prevents RNA from being bound and degraded by the RNA degradosome (Figure 1B). Crystallography, site-directed mutagenesis and interactomics approaches revealed the novel structure of Dip (PDB ID 5FT0 and 5FT1) and identified the RNase E interaction site as the outer surface of the Dip dimer. Dip is able to hijack the RNA binding sites of the RNase E scaffold domain via extensive, acidic patches on its outer surface. A double mutation within the acidic surface patch was shown to abolish the interaction with RNase E *in vitro* (by electrophoretic mobility assays) and retained the wild type phenotype of *P. aeruginosa* when overexpressed *in vivo*. This suggests that multiple amino acids in the acidic surface patch contribute to the global interaction between Dip and the RNase E scaffold domain. Two bacterial regulatory factors, RraA and RraB have been found to control RNase E

activity in a similar way in *E. coli*. Both of these regulators bind the C-terminal domain of RNase E, but only RraA does so by occluding the RNA binding sites.[17] However, there is no structural similarity between RraA and Dip.

With the current crystallographic data, it is tempting to speculate that Dip is capable of assembling into a higher order structure when bound to RNase E. Such an oligomer of Dip may mimic an RNA duplex strand to misguide the *P. aeruginosa* RNA degradosome. This 'nucleic acid-mimicking' strategy would not be surprising as it has already been detected in other phages. For example, the Ocr protein of coliphage T7 mimics B-form DNA to hijack bacterial restriction enzymes and thereby protects T7 genomic DNA.[18] Current efforts are concentrating on structural approaches to elucidate the interaction model.

Potential therapeutic applications to treat bacterial infections inspired by Dip

The overuse of antibiotics has led to an ever increasing number of multidrug resistant bacteria since 2000.[19] Due to its remarkable capacity to withstand antibiotics, *P. aeruginosa* has joined the ranks of these 'superbugs'.[20][21] Since phiKZ uses Dip to inhibit the RNA degradosome in a direct and efficient way, it is tempting to apply the targeting of the degradosome in a similar manner as part of a new antibacterial strategy. Even though Dip decreases the growth rate of *P. aeruginosa* and *E. coli*, it does not kill these bacteria. Therefore, it should be evaluated whether the RNA binding segments of the RNA degradosome would be a good antibacterial target and if the action of Dip on these specific RNA binding sites can be mimicked by small molecules. However, given that previous successful efforts have been made to identify compounds to target the catalytic domain of RNase E in *E. coli* and *Mycobacterium tuberculosis* [22], it may be worthwhile to develop Dip-based small molecule inhibitors against the scaffold domain of RNase E to complement the catalytic domain inhibitors.

Although it remains to be seen whether Dip-based molecules can be effective inhibitors, the real strength of Dip may lay in its broad interaction range rather than in its toxicity.[10] It has been shown that in addition to being able to inhibit *P. aeruginosa* RNase E, Dip can inhibit the *E. coli* degradosome as well. Moreover, interactions with the RNA degradosome of the distantly related *Caulobacter crescentus* were detected *in vivo*. Therefore, Dip based inhibitors

could be tested against a series of pathogens, in isolation and in conjunction with the small molecule inhibitors against the catalytic domain.

In addition, it is conceivable that heterologous expression of Dip might even improve the infection of a series of designer phages for species like *P. aeruginosa*, *E. coli* and *C. crescentus*, by protecting phage mRNA and increasing expression efficiency during the infection cycle. The ability to generate designer phage with boosted virulence may be particularly appealing to the field of phage therapy, which currently uses natural phage to treat, amongst others, *P. aeruginosa* infections in severe burn wounds.[23][24]

Biotechnological applications of Dip

Since Dip can interact with the RNase E of several bacterial species and can inhibit RNA degradation without killing the bacterial cell, several possible biotechnological applications can be envisaged. Dip may have potential applications in improving recombinant protein expression in bacteria by stabilizing the mRNA of the recombinant protein *in vivo*, by co-expression of Dip (or addition of a small-molecule inhibitor). Also, Dip could be used as a protein additive in total RNA extraction kits to help stabilize RNA. In both examples, the dose and timing of Dip or small molecule application would need to be optimized to obtain a maximal yield, since RNase E plays important roles in RNA processing as well as degradation.[26]

In addition, there is great research interest in identifying specific enzymes responsible for the degradation or processing of RNA targets, e.g. when testing stress responses of bacteria. A specific example is that of the degradation of stable RNAs, rRNA and tRNA, upon starvation. Although RNase E is involved in the maturation of rRNA and tRNA, the contribution of the degradosome in the degradation of these stable RNAs remains underexplored.[27][28][29] Heterologously expressed Dip or Dip-based small molecule inhibitors could be employed to specifically lower the activity of the RNA degradosome, and thus facilitate such experiments. Presently, such experiments are typically performed using a strain of bacteria with a temperature sensitive RNase E gene product, since knock outs are non-viable. Raising the bacterial culture to a non-permissive growth temperature for the RNase E mutant strain will effectively result in inhibition of RNase E activity, however the change in temperature could also result in multiple

undesired and unrelated heat shock responses in the bacterial cell. Although RraA inhibits the degradosome activity via the same mechanism as Dip, It was shown that RNase E has a higher affinity for RNA than for RraA. [30] Dip on the other hand, is able to displace bound RNA from RNase E and is therefore believed to have a higher affinity towards its target than RraA.

Finally, Dip or Dip-based small molecule inhibitors could potentially be used in conjunction with CRISPR-Cas editing or RNA interference applications. Both the RNA interference and CRISPR-Cas mechanisms use short RNA fragments in association with a ribonuclease (complex) to target RNA or DNA substrates in the cell. In the case of CRISPR-Cas for example, one may want to use a CRISPR array to knock out several genes at once. The corresponding pre-crRNA will be long and could be subject to degradation by the bacterial RNase E. In antisense RNA experiments, on the other hand, the specificity of the antisense transcript increases with its length. However, long antisense RNAs are unstable and might be subject to degradation by ribonucleases. Therefore Dip or Dip-based small molecule inhibitors might improve the performance of CRISPR-Cas and long antisense RNAs *in vivo* to knock down targeted gene expression.

In conclusion, these data indicate that molecular phage-bacteria interactions continue to reveal novel mechanisms of metabolism regulation as well as unique protein structures which can inspire application-driven biotechnological developments.

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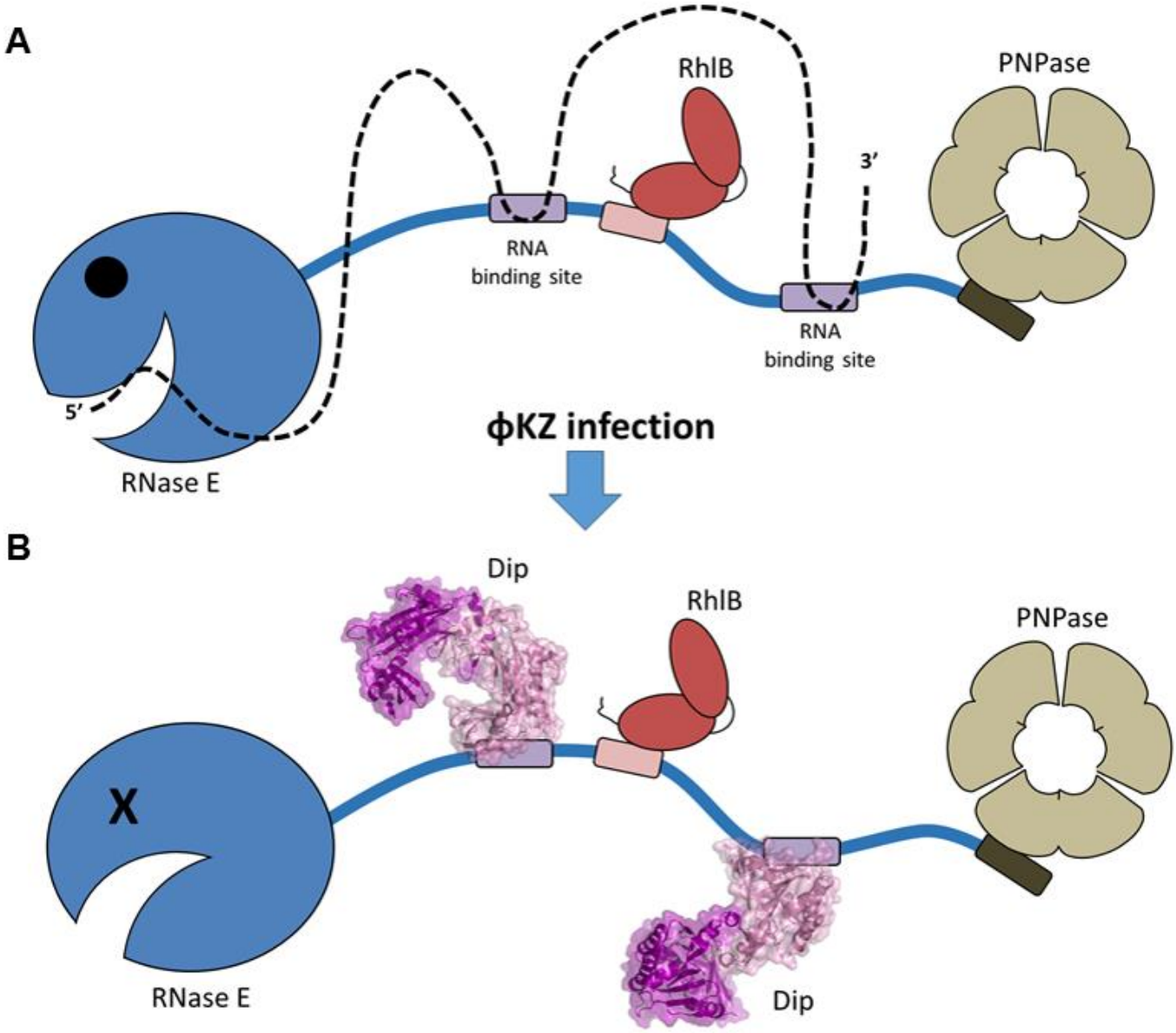

Figure 1. Displacement of RNA from the RNase E scaffold domain by Dip. (A) global composition of the P. aeruginosa RNA degradosome as observed in pull down assays. In the absence of Dip, RNA (dotted line) is bound by the RNase E scaffold domain and degraded by the catalytic domain.

(B) When Dip is present, the RNase E scaffold domain is hijacked and RNA is no longer degraded.